



Direct and simultaneous determination of reduced and oxidized glutathione and homogluthathione by HPLC-ESI/MS (TOF) in plant tissue extracts

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Introduction

Glutathione and homogluthathione as well as ascorbic acid, are low-molecular mass antioxidants involved in cellular redox homeostasis in plants (Noctor and Foyer, 1998). Efforts are being made to develop methods to determine the concentrations of oxidized and reduced forms of these compounds and their ratio (Camera et al, 2002). Many of the methodologies developed so far, however, are time-consuming and complex, and therefore analytes can decompose and/or their redox status can change during the analysis process. We have developed a method that allows for the simultaneous determination of reduced (GSH and hGSH) and oxidized forms (GSSG) of these compounds, and is also suitable for the determination of ascorbic acid (ASA) and S-nitroglutathione (GSNO).

Experimental

-Tissue extraction was done using 5% metaphosphoric acid (MPA) essentially as described by Davey et al (2003). All extraction steps were done at 4°C and under green safelight in order to minimize ASA degradation. Isotopically labeled ASA (ASA*) and GSH (GSH*) were added as internal standards.

-HPLC-ESI/MS(TOF) analysis were done in a Waters Alliance 2790 HPLC (Waters) coupled to a BioTOF II Time Of Flight mass spectrometer equipped with an electrospray ionization chamber (Bruker Daltonics).

-Samples or standard solutions were injected on a RP-18e monolithic column and eluted with a 1 mL min⁻¹ linear gradient of 0,1% formic acid and acetonitrile. Electrospray conditions, such as drying gas and capillary voltage were adjusted to maximize GSSG ionization.

-Different validation parameters such as calibration curves recovery, repeatability, limits of detection. Sample extracts from different plant tissues were analyzed. See Rellán et al. (2006) for further details.

Results

-Analysis were done in the negative mode as the signals were stronger than in the positive mode and ASA did not ionize in the positive mode. Mass spectra in the negative mode from the different compounds can be seen in Fig. 1.

-All analytes (GSH, GSSG, hGSH, ASA and GSNO) were base peak resolved in only 6 min. Isotopically labeled ASA, co-elute with ASA whereas isotopically labeled GSH eluted close to GSH. (Fig 2).

-Calibration curves were linearly fitted with r^2 between 0.991 and 0.998. LOD were in the range of 20-100 pmol. Repeatability measured as RSD of peak area was between 4 and 7% in the intraday test and between 7 and 13% in the interday test. RSD values for time retention were lower than 1% in both cases. Recovery obtained by spiking *B. vulgaris* leaf extracts with known amounts of analytes were in the range of 92 to 105%.

-Analyte concentrations in the different plant tissue extracts are presented in Table 1 and a chromatogram of *Prunus persica* leaf extract is showed in Fig. 3.

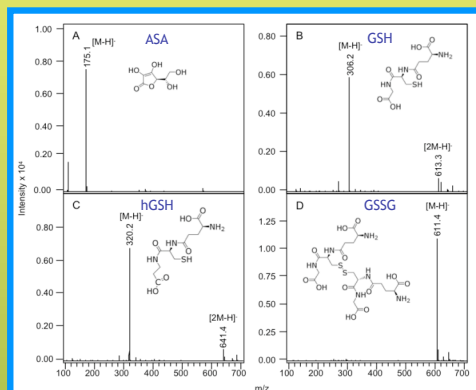


Fig 1. Negative ion mode ESI-MS(TOF) mass spectra and chemical structure of A: ascorbic acid, B: glutathione, C: homogluthathione and D: oxidized glutathione

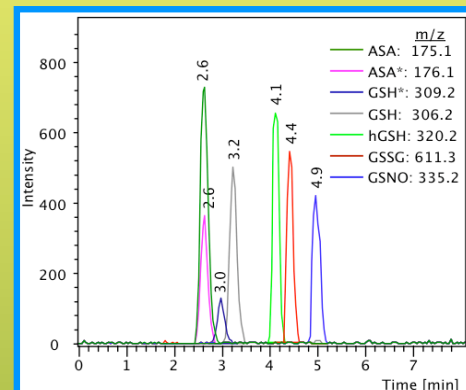


Fig. 2 Chromatogram of a standard solution of 200 μM ASA, 100 μM ASA*, 25 μM GSH*, 75 μM GSH, 75 μM hGSH, 25 μM GSSG and 75 μM GSNO in 2.5% MPA, 1 mM EDTA and 0.1 formic acid

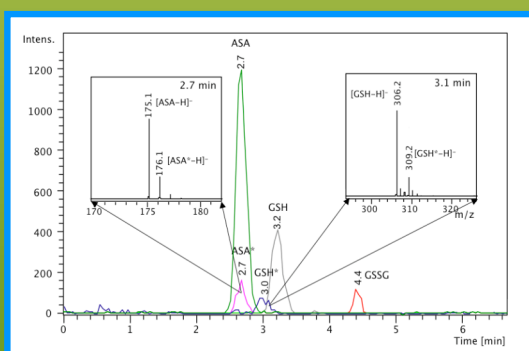


Fig. 3 Chromatogram of a field grown *Prunus persica* leaf extract, showing peaks corresponding to ASA (175.1 m/z), GSH (306.2 m/z) and GSSG (611.4 m/z) and to the internal standards ASA* (176.1 m/z) and GSH* (309.2 m/z). Insets show zooms of the mass spectra at 2.7 and 3.1 min

Plant tissue	GSH	hGSH	GSSG	ASA
	nmol g ⁻¹ FW			
<i>Medicago truncatula</i> (nodule)	202.8 ± 21.7	47.4 ± 14.7	7.0 ± 1.7	1,471 ± 54
<i>Medicago sativa</i> (leaf)	ND	1,333.3 ± 152.8	ND	3,523 ± 68
<i>Hordeum vulgare</i> (leaf)	187.5 ± 25.1	ND	22.9 ± 0.9	2,344 ± 51
<i>Lycopersicon esculentum</i> (leaf)	707.3 ± 54.4	ND	47.3 ± 5.5	4,370 ± 306
<i>Beta vulgaris</i> (root)	92.1 ± 14.8	ND	46.1 ± 10.1	ND
<i>Beta vulgaris</i> (leaf)	152.2 ± 8.8	ND	23.7 ± 0.2	2,240 ± 78
<i>Prunus persica</i> (leaf)	155.4 ± 9.5	ND	5.8 ± 0.1	8,730 ± 225

Table 1: Contents of GSH, hGSH, GSSG and ASA found in plant tissues using the HPLC-ESI/MS(TOF) method. Values are means ± SE (n = 3). (ND: not detected, below limit of detection, FW: Fresh weight)

Conclusions

-The method developed permits the direct and simultaneous analysis of GSH, GSSG and homologues, ASA and GSNO with extreme selectivity and good sensitivity and reproducibility.

-The rapidity of the analysis allows for a high analysis throughput. More than 75 samples per day can be analyzed.

-The high resolution of the MS (TOF) spectrometer used can give information on isotopic distribution (see insets in Fig. 3), allowing its use as a tool in metabolic studies with stable isotopes.

References

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